APPLICATION FOR LETTERS PATENT

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SUPERCRITICAL FLUID-ASSISTED NEBULIZATION
AND BUBBLE DRYING

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SUPERCRITICAL FLUID-ASSISTED NEBULIZATION AND BUBBLE DRYING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of Application No. 09/591,213, filed June 9, 2000, which claims the benefit of United States Provisional Application 60/138,394, filed June 9, 1999, which are incorporated by reference in their entirety to the extent not inconsistent with the disclosure herewith.

BACKGROUND OF THE INVENTION

With advances in gene therapy and recombinant DNA technology, protein pharmaceuticals are an important class of therapeutic drugs. For example, pulmonary delivery of therapeutic peptides and proteins has received significant attention in recent years, for the treatment of respiratory illness and as an attractive alternative to injection for the systemic delivery of macromolecules. However, the commercial production of protein pharmaceuticals is severely limited by chemical and physical degradation of the proteins which can lead to biological inactivation (Manning, M.C. et al. (1989), "Stability of Protein Pharmaceuticals," Pharm. Res. 6:903-918; Lai, M.C. and Topp, E.M. (1999), "Solid-State Chemical Stability of Proteins and Peptides," J. Pharm. Sci. 88:489-500). Many of these degradation processes use water for hydrolysis and/or other degradation pathways. Therefore, many protein pharmaceuticals are prepared in the solid state as dry powders to prolong the useable shelf life of the product and the storage stability of the product. Protein unfolding in the dried solid can lead to irreversible denaturation upon immediate rehydration and significant reduction of long-term storage stability.

Supercritical fluids are substances at a temperature and pressure above a critical temperature and pressure where the substance has a density, compressibility and viscosity intermediate between a gas and a liquid. Near-critical fluids are similar to supercritical fluids and are defined as fluids within 10% of the critical temperature and the critical pressure. For

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example, since the critical temperature of CO_2 is 31.6°C (304.6K) and the critical pressure is 1073 psi, CO_2 above 2°C (275K) and 966 psi is near critical. Supercritical fluids have been researched for their use in the production of fine powders of pharmaceuticals, however these technologies (supercritical fluid nucleation (Larson, K.A. and King, M.L. (1986), "Evaluation of

5   Supercritical Fluid Extraction in the Pharmaceutical Industry," Biotechnol. Prog. **2**:73-82), rapid expansion of a supercritical solution (Tom, J.W. and Debenedetti, P.G. (1991), "Precipitation of Bioerodible Microspheres and Microparticles by Rapid Expansion of Supercritical Solutions," Biotechnol. Prog. **7**:403-411) and gas antisolvent techniques (Randolph, T.W. et al. (1993), "Sub-micrometer-sized biodegradable particles of poly(L-lactic acid) via the gas antisolvent

10   spray precipitation process," Biotechnol. Prog. **9**:429; Meyer, J.D. et al. (1998), "Preparation and *in vitro* characterization of gentamycin-impregnated biodegradable beads suitable for treatment of osteomyelitis," J. Pharm. Sci. **87**:1149; Winters, M.A. et al. (1996), "Precipitation of proteins in supercritical carbon dioxide," J. Pharm. Sci. **85**:586; Palakodaty, S. et al. (1998), "Supercritical fluid processing of materials from aqueous solutions: the application of SEDS to

15   lactose as a model substance," Pharm. Res. **15**:1835) require that the pharmaceutical be soluble directly in the supercritical fluid or be precipitated by the supercritical fluid from nonaqueous solvents such as dimethylsulfoxide. The nebulizer system disclosed in U.S. Patent No. 5,639,441 (Sievers, R.E. and Karst, U., issued June 17, 1997) and divisional application 08/847,310 permits the use of mixtures of supercritical fluids with immiscible liquids such as

20   water to process substances that are not soluble in the supercritical fluid to form aerosols of vapors. Therefore, using the methods and devices disclosed in U.S. Patent No. 5,639,441 particles of water soluble proteins, excipients, stabilizers, bulking agents and/or surfactants may be formed rather than just particles of those compounds that are soluble in supercritical fluids and/or organic solvents. U.S. Pat. No. 5,639,441 is hereby incorporated by reference, to the

25   extent not inconsistent with the disclosure herein. Unlike the other precipitation methods, e.g., the SEDS process and GAS processes referred to above, no organic solvents are required in the new process; only the drug, water and the supercritical or near-critical fluid (for example, carbon dioxide) are needed.

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Even though particles of water soluble proteins and other aqueous formulations can be prepared, no method to form suitable dry powders of these proteins and/or formulations existed until now. Existing technologies to produce dry protein powders, such as spray-drying, freeze-drying, or ultrasonic nebulization, suffer from a variety of problems.   In general, dry protein

5   powders are often irrevocably inactivated when produced by prior art methods because the processing steps involved in these methods, temperature required to dry the proteins using these methods and dehydration processes of these methods damage the delicate structure of the protein. Also, for use in direct inhalation applications, powders must be small enough to allow for effective pulmonary delivery. Drug delivery via a pulmonary route is preferred over other

10   delivery routes such as injections for reasons such as decreased pain and delivery of the drug to the desired location more quickly. If the particles produced by the drying process are larger than desired, they must be jet-milled or mechanically ground. This creates an additional physical stress on the molecules and may impart a further loss of protein activity.   Dry powders produced in the correct size region could be used directly in dry powder inhalers for pulmonary delivery.

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Spray-drying is a currently-available method to produce dry protein powders. In the spray-drying technique, a jet nebulizer is used to form a plume of droplets. In one type of nebulizer, a liquid sample is sucked through a small diameter tube by a high-pressure stream of gas. The gas breaks up the liquid into fine droplets. The gas can also flow across the small

20   diameter tube at right angles and form droplets in a similar manner. Ultrasonic nebulizers use ultrasonic vibrations coupled to the sample solution that cause the solution to break up into small droplets. One disadvantage of the method of spray-drying is the plume of molecules exiting the jet nebulizer is not very dense. This results in a process that is slow in producing a desired amount of protein. Freeze-drying is another currently used method to produce dry protein

25   powders wherein aqueous solutions of drugs are frozen and placed under a vacuum to sublime the water. One disadvantage of the method of freeze-drying is the drying process is very slow. Also, the particles produced are relatively large, requiring additional processing steps to produce pharmaceutically desirable sizes.

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United States Patent 6,063,138 (Hanna, et al., issued May 16, 2000) and related EP 0767702 describes methods of forming particles of a substance by co-introducing a supercritical fluid; a solution or suspension of the substance in a first vehicle; and a second vehicle which is substantially miscible with the first vehicle and substantially soluble in the supercritical fluid into

5   a particle formation vessel which is maintained at supercritical pressure and temperature.

PCT published application PCT/US99/19306 (WO 010541) (Edwards et al.) describes methods of forming particles by combining a bioactive agent, a phospholipid and an organic solvent or organic-aqueous co-solvent to form a mixture which is then spray-dried.

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United States Patent 5,695,741 (Schutt et al., issued December 9, 1997) and related United States Patents 5,639,443 (Schutt et al., issued June 17, 1997) and 5,720,938 (Schutt et al., issued February 24, 1998) describe "microbubbles" useful for magnetic resonance imaging and ultrasound imaging. The "microbubbles" are prepared by spray-drying a liquid formulation to

15   produce microspheres having voids and then permeating the microspheres with a fluorocarbon gas osmotic agent.

United States Patent 5,928,469 (Franks et al., issued July 27, 1999) describes mixing materials with a carrier substance that is water-soluble or water-swellable and spray drying the

20   resultant mixture to form particles containing both the material and the carrier substance in which the carrier substance is in an amorphous (glassy or rubbery) state. Franks describes spray drying at gas temperatures of 100 to 300°C.

United States Patent 6,001,336 (Gordon et al., issued December 14, 1999) describes

25   spray drying suspensions of a hydrophobic component and a hydrophilic component dissolved in an aqueous solution.

United States Patent 5,851,453 (Hanna et al., issued December 22, 1998) and related EP 0 706 421 describe a method and an apparatus for forming particulate products by introducing a

30   supercritical fluid and a solution or suspension of a substance in a vehicle soluble in the

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supercritical fluid into a vessel which is maintained at controlled temperature and pressure. WO 95/01324 (York et al., published January 12, 1995) describes particles of salmeterol xinafoate using this method.

5 WO 99/16419 (Tarara et al., published April 8, 1999) describes preparing "perforated microstructures" by atomizing a liquid and spray drying the liquid droplets that are formed. WO 00/00215 (Bot et al., published January 6, 2000) describes delivery systems of "perforated microstructures" containing "bioactive agents" .

10 WO 99/59710 (Hanna et al., published November 25, 1999) describes a method and apparatus for forming particles of a substance by dissolving or suspending the substance in a first vehicle which is or contains a first supercritical or near critical fluid and passing that solution or suspension into a particle formation vessel which contains a second supercritical fluid. The vessel is maintained at temperatures and pressures so that the second fluid remains supercritical.

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WO 98/36825 (Hanna et al., published August 27, 1998) describes a method and apparatus for forming particles by directing two supercritical fluids, one containing the substance of interest, into a heated and pressurized chamber.

20 There is a need for stable or pharmaceutically-active proteins in dry form, and a method to produce stable or pharmaceutically-active proteins in dry form. Also, there is a need to produce smaller particles with improved pharmaceutical activities.

BRIEF SUMMARY OF THE INVENTION

25 This invention provides a method for forming fine dry particles comprising:

(a) forming a composition comprising one or more substances and a supercritical or near critical fluid;

(b) rapidly reducing the pressure on said composition, whereby droplets are formed;

(c) passing said droplets through a flow of gas heated from about 2°C to about

30 300°C.

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Preferably bubble drying should be conducted at temperatures above ambient temperature and below 100°C to minimize degradation of the pharmaceuticals. Given sufficient residence time and dilution, a flow of dry gas will dry the fine droplets without external heating. Heating accelerates drying by increasing the vapor pressure of water. The composition may also comprise an aqueous solvent.

Also provided is a method of forming fine dry particles comprising:

(a) mixing an aqueous solution containing the substance of interest and a supercritical or near supercritical fluid, forming a composition;

(b) rapidly reducing the pressure on said composition, whereby droplets are formed;

(c) passing said droplets through a flow of gas heated from about 2°C to about 300°C.

Also provided is a method of forming fine dry particles comprising:

(a) equilibrating an aqueous solution of the substance of interest with a supercritical or near supercritical fluid, forming a composition;

(b) rapidly reducing the pressure on said composition, whereby droplets are formed;

(c) passing said droplets through a flow of gas heated from about 2°C to about 300°C.

Also provided is a device for forming fine dry particles, consisting essentially of:

(a) a first pressurized chamber containing a first nongaseous supercritical or near critical fluid;

(b) a second chamber containing a solution or suspension of a substance in a second nongaseous fluid;

(c) a mixing chamber for mixing said solution or suspension and first fluid connected to said first and second chambers by conduits;

(d) first flow control means connected to the conduit between the first chamber and the mixing chamber for passing said first fluid into said mixing chamber;

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(e)   second flow control means connected to the conduit between the second chamber and the mixing chamber for passing said second fluid into said mixing chamber;

(f)   a restrictor connected to said mixing chamber for conducting the composition out of the mixing chamber into a rapid expansion region having a pressure below that of the supercritical or near critical fluid where a dispersion of fine particles of said substance is formed;

(g)   a drying chamber connected to the restrictor;

(h)   a source of gas connected to the drying chamber at one or more inlets;

(i)   means for collecting particles after they pass through the drying chamber.

The mixing chamber is preferably a low dead volume tee.

Fine particles are those with diameter less than about 5 micrometers. Particles formed by the methods of the invention may vary in diameter between about 0.1 micron to about 5 microns. The particles produced may be smaller than 0.1 microns, but current detection methods are size limited in the lower size range, and small particles do not constitute a significant fraction of the mass. The particles may be of any distribution of diameters. For certain applications, for example inhalation therapy, it is preferred that most particles be within the respirable range for delivery to the deep lung alveoli. Preferably the particles range in size from 1 to 3 microns for inhalation applications. Particles may be different sizes for other applications, as known to the art or readily determined without undue experimentation. It is preferred that for inhalation applications, the particles have a small variance from the average size.

As used herein, "dry" or "dried" include particles that include some moisture, preferably not more than 5% by weight Dry particles include those particles which include from 0.0001% to 1% moisture, from 1% to 3% moisture, from 1% to 5% moisture, from 5% to 10% moisture, and combinations of those ranges.

Particles of various shapes are included within the invention. For example, particles may be hollow, or "bubbles". Bubbles are hollow-centered, similar to a tennis ball or a ping pong

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ball, although they may not be as spherical as a tennis ball or a ping pong ball. The diameter of the particle is typically about 10 to 10,000 times the thickness of the skin. Other particles formed by the method of the invention are not hollow. Higher drying temperatures (~100°C) favor forming hollow particles, while the same substance may give solid spheres if dried slower

5   at lower temperatures.

"Composition" does not mean all substances are necessarily soluble in each other. Substances which may be made into particles by the methods of the invention include any substance which is either soluble in a supercritical fluid or near critical fluid or mixtures thereof; 10   or a substance which is soluble or suspendable in an aqueous solution. The aqueous solution may also include various co-solvents, but avoiding the use of organic solvents may have environmental and toxicological benefits. Some substances which may be made into particles include: a physiologically active composition comprising one or more substances selected from the group consisting of surfactants, insulin, amino acids, enzymes, analgesics, anti-cancer agents, 15   antimicrobial agents, viruses, antiviral agents, antifungal pharmaceuticals, antibiotics, nucleotides, DNAs, antisense cDNAs, RNAs, peptides, proteins, immune suppressants, thrombolytics, anticoagulants, central nervous system stimulants, decongestants, diuretic vasodilators, antipsychotics, neurotransmitters, sedatives, hormones, anesthetics, anti-inflammatories, antioxidants, antihistamines, vitamins, minerals and other physiologically active 20   materials known to the art. Particles of monoclonal antibodies and vaccines may be produced. Particles of substances such as sodium chloride may also be produced. Some substances may be pharmaceutically-active. "Pharmaceutically-active protein" indicates that the protein has sufficient activity so as to be pharmaceutically useful. Other substances may not be physiologically or pharmaceutically-active.

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Various additives may be used in the methods and particles of the invention. These additives may be added to the substance of interest or any solvent used in the process. Additives may also be added directly to the particles after formation. Additives include stabilizers, excipients, bulking agents and surfactants. The use of stabilizers in protein formulations protects 30   against loss of protein activity upon drying. Stabilizers include, without limitation, sugars and

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hydrophilic polymers, such as polyethylene glycol, hydroxy ethyl starch, dextran or others. The stabilizer is preferably a sugar or mixture of different sugars. Sugars that can be used include mannitol, sucrose, lactose and trehalose, and other mono-, di-, and oligosaccharides. Addition of one or more stabilizers to the protein solution prior to dehydration significantly inhibits the

5   conformational changes within the protein that are believed to be linked to a loss of enzymatic activity. One or more surfactants can be added to alleviate stresses between droplet/air interfaces and decrease degradation that may occur upon drying. Surfactants may be added to alleviate agglomeration or clumping that may occur upon drying. Surfactants are also thought to assist in the formation of spherical particles. Examples of surfactants that may be used include:

10   polyoxyethylene (20) sorbitan surfactants (Tweens), such as Tween 20, Tween 40, Tween 80 and Tween 85; stearic acid; myrj (PEG monostearates), Span 85 (sorbitan trioleate) and polyether-carbonate block co-polymers of the type reported in Beckman et al. (2000) Nature **405**:165. Phospholipids, including phosphoglyceride may be used. Surfactants and other agents, such as guanidinehydrochloride, may facilitate protein refolding, coupled with pressure treatment. (St.

15   John, R.J. et al. (1999) Proc. Natl. Acad. Sci. **96**:13029). Bulking agents and excipients may be inert or active.

If needed for stability of the final formulation, buffer is preferably added first. If more stability is required, sugar may be added. If still more stability may be added, surfactant may be

20   added. A pH buffering substance is useful to counteract the rapid drop in pH from carbon dioxide dissolution at high pressure to form carbonic acid.

For low potency drugs, the fraction of additives in the powder should be minimized; for high potency drugs, inactive excipients (diluents) sometimes constitute more than 99% of the

25   mass. Additives may be added in any useful amount to the composition. Additives are typically used at a concentration of between about 0.001 to 0.5 wt% of surfactant (preferably between about 0.001 to 0.1 wt%), and between about 0.05 to 25% of stabilizer (preferably, when sugar is used, between about 0.1 to 20%, limited by the solubility limit of sugar) by weight to a solution comprising a protein of interest. These percentages are expressed for the aqueous solution

before spraying and drying. The final percentage of sugar in the dried powder can be as high as ~99.9%.

The composition may comprise a substance which is soluble in the supercritical or near critical fluid, such as a lipophilic compound. A mixture of supercritical or near critical fluids may be used. The composition may also comprise an aqueous solution or suspension of the substance and a supercritical or near critical fluid (or fluids). Droplets of substances which are not soluble in the supercritical or near critical fluid, such as hydrophilic substances, are then formed. In one embodiment of this method, an aqueous solution comprising a dissolved or suspended compound is pumped into a low-dead volume tee while a supercritical or near critical fluid is pumped into another leg of the tee. The resulting emulsion or supercritical solution pressurized to a pressure near or above the critical pressure of the supercritical fluid (about 70 to about 100 atmospheres when carbon dioxide is used) is allowed to expand to atmospheric pressure out a flow restrictor, forming fine droplets or bubbles containing the dissolved drug species. This aerosol is directed into a drying chamber where solvent evaporation and particle formation takes place.

A second variant of this method allows aerosols to be formed without a low-dead volume tee. In this method, an aqueous solution of precursors is first allowed to equilibrate with a near critical or supercritical fluid in a static canister or chamber, preferably with stirring or agitation. This composition is then allowed to expand to atmospheric pressure out of a flow restrictor, forming fine droplets. These droplets are directed into a drying chamber where solvent evaporation and particle formation takes place.

The initial concentration of the substance of interest in either the supercritical fluid or the aqueous solvent (or solvent mixture) is limited only by the solubility or saturation point of the substance in the solvent or supercritical fluid. Typical starting concentrations are about 1% to about 25% w/w of total solids in the solution or fluid.

Preferably the supercritical fluid is carbon dioxide because carbon dioxide is endogenous and relatively non-toxic, as well as having a critical pressure and temperature easily obtainable. Other supercritical or near-critical fluids may be used, provided that the critical temperature and pressure are obtainable and useful. Carbon dioxide is currently less expensive than any organic

5   solvent and its use avoids VOC emissions. Carbon dioxide's solubility in water is about 2% at 100 atm at near-ambient temperatures.

A number of fluids suitable for use as supercritical fluids are known to the art, including carbon dioxide, sulfur hexafluoride, chlorofluorocarbons, fluorocarbons, nitrous oxide, xenon,

10   propane, n-pentane, ethanol, nitrogen, water, other fluids known to the art, and mixtures thereof. The supercritical fluid is preferably carbon dioxide or mixtures or carbon dioxide with another gas such as fluoroform or ethanol. Carbon dioxide has a critical temperature of 31.3 degrees C. and a critical pressure of 72.9 atmospheres (1072 psi), low chemical reactivity, physiological safety, and relatively low cost. Another preferred supercritical fluid is nitrogen.

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The gas that contacts the flow of the droplets is preferably inert, and a preferred embodiment is nitrogen gas, but the gas may be chosen so as to react with the molecule of interest in the course of drying. Preferably the flow of gas contacting the flow of the sample forms a sheath surrounding the flow of the sample, but the flow of gas may contact the flow of

20   the sample by other means such as turbulent mixing. Preferably this gas is heated to a temperature sufficient to cause the desired level of particle drying and also not substantially degrade the biological activity of particles. The gas may be heated to between about 2°C to about 300°C, preferably below 100°C, although depending on the substance being dried and the constituents of the composition, the temperatures may be adjusted. A preferred range of drying

25   temperatures is between about 35°C to about 100°C.

Preferably the gas is contained in a drying chamber such as a drying tube. The drying tube is as long as necessary to produce particles having the desired level of moisture by the time the particles reach the end. The drying tube is preferably larger than the diameter of the droplet

30   plume formed from the rapid expansion. The drying tube may be heated, but that is not required.

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Preferably most of the heat required for vaporizing water is provided by heating the drying gas before it enters the drying chamber. Heating the drying tube may assist in preventing condensation on the surfaces of the tube. The drying tube may be heated externally by means of a lamp such as an infrared lamp, or internally by any means known in the art such as a heating wire imbedded in the material making up the tube. The drying tube may be made from any suitable material which can withstand the temperatures to which it is subjected. Examples of material from which the drying tube can be made are stainless steel or borosilicate glass. Any other design of drying chamber may be used if the desired results are obtained. Other apparatuses, for example a microwave oven, may be used in place of the drying tube to perform the same function.

Rapid reduction of the pressure of the composition is typically performed using a flow/pressure restrictor. The restrictor may be a hollow needle of an thermally conductive material such as stainless steel or a ceramic material, or other material which is able to withstand the pressure and temperature placed upon it. The restrictor may alternatively be a fused silica flow restrictor, or a ceramic multi-channel bundle of capillaries such as that discussed in more detail elsewhere. Also, high pressure sintered stainless steel filters may be used to generate aerosols. The length of the restrictor is typically about 2 inches long; however, the length must not be so long as to cause low flow rates or the precipitation of sufficient solid substance in the restrictor to cause clogging. The restrictor may have as large a diameter as desired, as long as the desired size particles are formed and the pumps have sufficient capacity to maintain the pressure. The lower limit of diameter is determined by the viscosity of the solution being passed through the restrictor. If the viscosity is too high, particles are not formed.

The invention also provides a multichannel restrictor. These openings may be spaced approximately the same distance from each other. The structure may have a cylindrical, a hexagonal, or other shape which allows it to be coupled with the other components used. The openings may be any suitable shape, such as round or hexagonal. An embodiment of one such structure has about 900 non-concentric parallel channels in about a 2 mm total diameter. This multichannel restrictor may have a total diameter which provides the desired particle formation.

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Preferably each channel has an inner diameter between about 40 µm and about 125 µm. Other multichannel structures may be used, and the openings do not need to be a similar size, although it is preferred if the openings are similarly sized.

5   The exit tip of the restrictor structure may be flat or substantially flat, or may be shaped. One shape that is particularly useful is formed by removing material from the sides of a flat end, to form an elongated point, similar to a pencil. This modification gives a more dispersed stream of droplets emitted over a 180° angle which is useful to assist in preventing agglomeration of particles as they undergo bubble drying. The particular geometry which gives the best results,

10   depending on the results desired, may be discovered by routine experimentation. Any other means available for reducing the pressure on a composition may be used to accelerate drying.

With many openings through which the composition may pass, the flow rate through the system may be increased and the throughput of the system increased. The overall throughput

15   rate is principally controlled by the total inner diameter of the flow restrictor. Various inner diameters of single channel restrictors may also be used, including 75 micron, 100 micron, 170 micron, and 200 to 1000 micron. Another benefit of multi-channel restrictors over single channel ones is that if one channel becomes clogged, the remainder of the channels remains functional.

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The method of this invention may be used for processes in which faster drying than currently available is desired. Fast drying may occur because the swelling/bursting processes of the invention gives greater surface area for drying, although applicants do not wish to be bound by this theory.

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Various particles including pharmaceutically-active protein compositions in dry form comprising particles of a protein of interest and optionally containing one or more additives selected from the group consisting of excipients, stabilizers, bulking agents and surfactants, wherein the additives are present at a concentration of about 0.001% to about 99.9%, measured

30   by weight of the dry protein, and wherein the particles have diameters of about 0.1 microns to

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about 10 microns are produced by the method of this invention. Particles may have a variety of bulk densities depending on the particular substances involved and the conditions under which particle formation and drying occur. For example, particles with bulk densities of between about 0.1 and 1.5 g/cm^3 may be formed. The bulk density may be less than 1 g/cm^3, less than 0.8 g/cm^3, less than 0.5 g/cm^3, less than 0.4 g/cm^3 or other ranges. Particles may have a variety of activities after rehydration. For example, dry particles with at least 90% of the original activity upon rehydration are included in the invention. Particles with 90-95%, 90-100%, 100-120% original activity are also among those included in the invention.

The particles may be stored in any convenient manner after formation and drying, including placing in bags or other storage devices or additional drying during storage over desiccants such as P_4O_{10} can be undertaken.

The invention also provides a nebulization system using an injection port that requires a lower volume of sample than currently available systems. This is an advantage when conducting laboratory scale experiments, for example, on costly samples. The injection port also permits equilibration of the nebulization and drying system with the solvents only, followed by introduction of a solution comprising the protein of interest after the system has been equilibrated. This reduces waste of the protein. This nebulization system may be attached to a bubble drying system, or may be coupled with equipment used in conventional spray drying to permit faster drying at lower temperatures.

A method of making droplets by injecting a volume of one or more substances into a flow of a solution comprising an aqueous or supercritical or near supercritical solution using a means for introducing a small sample volume into the flow and subjecting the resulting solution to a rapid pressure decrease to form droplets is provided. Preferably the introducing means is an injection port, such as that used for HPLC. Preferably the volume introduced is about 0.01 mL to about 10 mL.

A method of delivery of proteins or other substances comprising: drying a protein or other substance using the method of this invention, reconstituting the protein with water or other suitable substance, and delivering via desired means is provided.

5   Stable and/or pharmaceutically active particles are also provided. "Stable" means resistant to decomposition during storage, shipping, reconstitution, and administration.

A device for rapid expansion of a composition comprising a low dead volume tee through which said composition passes and a restrictor with more than one substantially parallel non-
10   concentric channels affixed to said tee is also provided. A low dead volume tee is a mixing tee having a volume of about 0.2 to 10 µl. The tee may be affixed to the restrictor by any suitable means, for example, epoxy or appropriate fittings.

The drying technique of the invention has advantages over conventional drying
15   techniques. The drying technique of the invention is scalable without significant alteration of particle size or morphology. The method also provides particles having lower density than particles produced by other methods. This leads to particles with small aerodynamic sizes, but large absolute particle dimensions, since in one embodiment, additional gaseous supercritical fluid is formed and leaves the substance of interest containing particles with porous or hollow
20   structures. This permits larger particles with lower momentum to reach the deep lung than would otherwise be possible, in one application.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 is a schematic diagram of a supercritical fluid assisted nebulization and bubble
25   drying system.

Figure 2 is a diagram of a drying tube used in the invention.

Figure 3 is an electron micrograph of the end of a multi-channel flow restrictor.

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Figure 4 is an electron micrograph of a shaped multi-channel flow restrictor.

Figure 5 is a graph of the buffering capacity of monobasic/dibasic potassium phosphate (♦, pH7), Tris/Tris HCl (▲, pH 7.2), citric acid/sodium citrate (■, pH 5.5), and acetic acid/sodium acetate (●, pH5) upon nebulization with supercritical CO_2.

Figures 6A - 6G are electron micrographs of dry protein powders produced by the method of this invention.

Figure 7 shows electron micrographs of lysozyme powders produced by the method of this invention with "Buffer only" powder (A) 10% mannitol (B), 10% sucrose (C), and 10% sucrose with 0.01% Tween 20 (D).

Figure 8 shows X-ray diffraction patterns for samples prepared by supercritical CO_2-assisted nebulization of 4 mg/mL lysozyme, 100 mM phosphate buffer at pH 7.0 and 10% mannitol (A) or 10% sucrose (B), with final approximate solid weight percentages in the bubble dried powders of 4% protein, 16% buffer and 80% sugar.

Figure 9 shows FTIR spectra of lysozyme powders produced by the method of this invention with (A) lysozyme in the native aqueous state; (B) lysozyme containing 10% sucrose and 0.01% Tween 20; (C) lysozyme with 10% sucrose; (D) lysozyme with 10% mannitol; and (E) lysozyme with buffer only dried at 70°C.

Figure 10 is a graph of the lysozyme enzymatic activity of rehydrated powders, produced using supercritical CO_2-assisted nebulization and bubble drying, compared to the activity of the original formulations.

Figure 11 is a graph of the activity of lactate dehydrogenase (LDH) powders produced by the method of this invention after rehydration.

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Figure 12 is a graph of the recovery of lysozyme activity using different excipients upon rehydration using different percentages of excipients.

DETAILED DESCRIPTION OF THE INVENTION

5   The advantages of the present invention over currently used techniques to form dried proteins include the ability to form stable formulations of a variety of different proteins and other materials that are currently unsuitable for drying using presently-available techniques. The present invention imparts lower stress and less severe damage to the substance during drying. Also, the fine powders of stable formulations including proteins formed by this method are

10   prepared directly in the inhalable size region, eliminating the need for additional mechanical micronization which could impart additional stresses to the dried formulation and further loss of activity. The temperatures preferably used in the process of the current invention are much lower than that of conventional drying processes. This decreased temperature of drying results in less extensive thermal degradation. The powders formed by the method of the current

15   invention may retain less water in the prepared powder than with conventional methods, eliminating the need for an additional drying step. The present invention reduces the amount of denaturation and resulting aggregation that is seen with conventional nebulization.

          One specifically exemplified embodiment of the present invention is shown in Figure 1

20   illustrating the flow of fluids, solutions and particles in the method of this invention. A carbon dioxide reservoir 10 (which can be a gas cylinder or other reservoir) containing liquid carbon dioxide is connected to a carbon dioxide pump 15 (which can be a syringe pump or other suitable pump) via conduit 70. The carbon dioxide pump 15 is connected to mixing tee 20 by means of conduit 71, through which the carbon dioxide is pumped under conditions at which it becomes a

25   supercritical fluid or near critical fluid, when it reaches the heated mixing tee 20. Aqueous solvent reservoir 40 is connected via conduit 72 to fluid pump 35, preferably a high performance liquid chromatography (HPLC) pump, which is connected via conduit 73 to the injection port 30.
          The injection port 30 is used to add an aqueous solution of protein or other drug, buffer, bulking agent, excipient, stabilizer and/or surfactant. The solution of aqueous solvent from reservoir 40,

30   and protein solution, with or without additives injected through injection port 30, is connected

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via conduit 74 to mixing tee 20. Mixing tee 20 preferably has a low dead volume, e.g., less than about 10 µl so than an intimate mixture of the supercritical carbon dioxide and the aqueous solution may be formed therein. Mixing tee 20 is equipped with pressure restrictor 25 to maintain back-pressure in mixing tee 20, and may be optionally equipped with heating coils to maintain

5 supercritical temperature therein. Upon passage of the resulting mixture from mixing tee 20 through small diameter pressure restrictor 25, sudden release of the pressure at the exit of the orifice of pressure restrictor 25 occurs and a very fine aerosol of solution droplets and/or bubbles is formed (80). The restrictor may comprise a single outlet, or may consist of many outlets. Aerosol 80 is then directed into the center of drying tube 45. Drying tube 45 is preferably less

10 than 1 meter long and 10 cm in diameter. Nitrogen gas from nitrogen reservoir 30 is added to drying tube 45 via conduit 85 through input ports 35. Preferably a plurality of input ports 35 are arranged equidistant around the center of drying tube 45. Lamp 90 may be used to heat drying tube 45. Nitrogen gas may also be heated as shown in Figure 2. Heated air or mixtures of gases may be used if there is no explosion hazard. Heat aids in the drying process and keeps water

15 from condensing on the inner walls of the tube and on the filter paper. If the water condenses on the filter paper, it may cause clogging. The output of drying tube 45 is fitted with filter paper holder 50 which is connected to vacuum pump 55 through a suitable connector 91.

In operation, the liquid carbon dioxide is pumped by means of supercritical carbon
20 dioxide pump 15 from carbon dioxide reservoir 10 via conduit 70 through pump 15 and via conduit 71 to the low volume (0.2 to 10 µl) mixing tee 20 where it becomes a supercritical fluid (if it is not already), or a near critical fluid. Pump 35 pumps aqueous solvent from solvent reservoir 40 via conduit 72 where it is pumped via conduit 73 to injection port 30, where the protein of interest is added as an aqueous solution. Additives may also be introduced, either

25 through injection port 30 or to the solvent in reservoir 40. Buffers may be used in the protein solution or aqueous solvent in reservoir 40 to reduce the effect of the possibility of denaturation of the protein and reduce the production of aggregates that may occur due to the otherwise uncompensated pH change due to the introduction of carbon dioxide in the mixture. Mixing tee 20 may be heated by the use of heater coils, and/or restrictor 25 may be heated to maintain the

30 temperature above the critical temperature of the carbon dioxide. The flow of carbon dioxide

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and aqueous solution are adjusted independently by means of valves, not shown in Figure 1. Flow rates can also be controlled by altering pumping conditions. The mixture in mixing tee 20 expands downstream and forms aerosol 80 comprising fine particles of the substance dissolved or suspended in the aqueous solution. The particles are directed in the center of drying tube 45

5 where they are dried and collected on filter paper in filter paper holder 50. Alternatively, particles may be collected using a cyclone separator or cascade impactor, for example, if the particle size distribution is suitable.

The use of injection port 30 allows equilibration of the system prior to introduction of

10 protein and/or additives. Equilibration of the system is reached when the temperature of the drying tube is equilibrated as measured by a thermocouple and the flow rates of aqueous solution and carbon dioxide reach steady states. After equilibration, a known volume of the protein formulation into the aqueous feed line may be injected through injection port 30.

15 A six-port injection valve may be adapted for use in a nebulization system so that in the load stage, there are two separate solution loops. Solvent is pumped from an HPLC pump through the mixing tee in one loop and the sample is contained within a sample loop, for example a 3 ml sample loop, that is not connected with the solvent loop in the load stage. During the inject stage, solvent is pumped from the HPLC pump through the sample loop and to

20 the mixing tee. This modification has the advantage of not pumping protein or other substances of interest through HPLC pistons and allows the ability to inject very small samples. The amount of protein required per run and the overall run times are substantially decreased.

When a sample injection valve is used, typical system parameters include: carbon dioxide

25 pressure 100 atm; carbon dioxide flow rate 0.3 ml/min; aqueous flow rate 0.3 ml/min, using a 5 cm long fused silica restrictor with 50 μm inner diameter.

Figure 2 shows a more detailed view of the drying tube and drying process. The solution from mixing tee 20 is passed through restrictor tip 70 into drying tube 45 through inlet 100,

30 where droplets are formed. Nitrogen (or other gas, either inert, or containing a substance that is

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desired to react with the aerosol particles) from reservoir 30 is passed via conduit 85 to gas drying column 60. The gas is then passed via conduit 95 to heating coil 65. In a preferred embodiment, the gas is heated to around 70°C and is present at a flow rate of around 15 L/min. Heated gas is passed through conduit 100 to drying tube 45 via gas inlets 35. In a preferred

5   embodiment, drying tube 45 is a glass tube with 5 input ports, one in the center where the aerosol is added, and 4 other inlets arranged around the center port. Lamp 90 is also used to heat drying tube 45 externally. At the output of drying tube 45, the particles are collected on filter paper held in holder 50.

10   Other configurations of drying tube 45 may be used. For example, more or fewer inlet ports 35 may be used. The gas may also be added to the center of the tube, and the particles added around the gas. Gas and particles may also be mixed together in the drying tube. The interaction of the drying gas and particles in the tube affect the characteristics of the final product.

15

Preferably all high pressure parts are made from stainless steel. Other inert materials or coatings may be used. Preferably filter paper holder 50 is made from stainless steel. The restrictor length is preferably about 2 in. (5 cm). The flow rates for the aqueous solutions in the above apparatus are about 0.5 ml/min to about 3 ml/min. If desired, the process may be

20   conducted on a larger scale or smaller scale by adjusting dimensions and flow rates while maintaining similar temperatures and pressures. In the specifically exemplified embodiment, flow rates of carbon dioxide and aqueous solvent are approximately 0.3 mL/min. when using a 50 μm inner diameter flow restrictor.

25   Injection port 30 allows several small volume aliquots (about 0.1 to about 10 ml) of protein formulations to be introduced in the same amount of time as required by one large volume (> 10 ml) in a system without the injection port. This permits several protein formulations with varying components and concentrations to be dried quickly with small amounts of protein used in each run. This is a significant advantage to commercial laboratory

30   scale spray driers which require up to 100 ml of protein solution per experiment. However, the

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method of the invention may also be practiced with larger volumes of proteins and with larger volumes of any substance desiring to be dried.

The restrictor may be a multichannel restrictor having a plurality of parallel tubes. One such multichannel restrictor may be fabricated from a glass multi-channel column (Alltech, Inc., Illinois). This column may be purchased in lengths up to 1 meter with about 900 holes through the length of the column each hole having an inner diameter approximately 40 or 50 microns or greater. A piece of such column can be used as a multichannel restrictor. One such product has a hexagonal shape (Alltech part number 17059). Other products have a round shape where the hexagon is placed in a circle of the same diameter. An electron micrograph of the end of such a restrictor is shown in Figure 3. The round shape restrictors are easier to attach to pumps and canisters with ferrule seals and Swagelok fittings and they can also be attached to stainless steel tubing or tee by applying epoxy pre-polymer mixture to the exterior of the glass or ceramic tubing and slipping it into a slightly larger steel tubing. The hexagonal voids can be filled with polymers or epoxys. These multichannel restrictors may be used in both static or dynamic processes. In a dynamic process, a low dead volume tee is used to intimately mix streams of liquid (near critical) or supercritical fluid such as carbon dioxide with an aqueous solution or suspension to be nebulized. In the static process, a solution of a substance of interest is pressurized by supercritical or near critical fluid at a temperature near its critical temperature. This allows some of the supercritical or near critical fluid (up to about 1 to 2 mole percent if carbon dioxide is used) to be dissolved in the water. When the solution approaches equilibrium, with mixing, if the aqueous solution and supercritical or near critical fluid are allowed to be ejected through a pressure restrictor, an aerosol is formed as the fluid exiting the pressurized fluid returns to atmospheric pressure or lower. The multichannel restrictor can also be used in the gas antisolvent methods, and any other methods where particles are desired.

The restrictor may have a shaped or substantially flat end. The end of a restrictor may be mechanically shaped. For example, part of the material may be removed using an abrasive. Some suitable abrasives include diamond-embedded nickel alloy pads, of the kind used by marble sculptors made by 3M, for example. Very small (micron sized) diamond crystals abrade

away the walls around each channel. The restrictor type shown in Fig. 3 after shaping with a diamond pad is shown in Fig. 4. Alternatively, fine frit silicon carbide "wet-dry" sandpaper can be used. The silicates in grout or tile can abrade and shape the restrictor. Diamonds are preferred.

5

Layers of the restrictor may also be etched away chemically. For example, selective layers of a glass restrictor may be etched with HF. One procedure that may be used is to force air in the tip slowly and dip the restrictor deeper and deeper into hydrofluoric acid (aq.). This will taper the end to a pencil-like tip point. Other suitable chemicals may be used. For example, if

10   the restrictor is metal, an acid such as hydrochloric acid may be used to remove layers. Combinations of mechanical and chemical etching may be used.

The angle of the conical tip may be varied from very acute to very sharp (from about 5 degrees from the plane of the body of the restrictor to about 89 degrees from the plane of the

15   body of the restrictor). The angle of the conical tip determines the volume in which the droplets will form, with a more acute angle increasing the volume at which the droplets will form. When the angle is more acute, the channel outlets appear as long ellipsoids, rather than round holes. Spraying over the area of a hemisphere (rather than in a single direction) allows droplets to be rapidly dispersed in the drying gas with less agglomeration.

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The benefits of using a multichannel restrictor include increased throughput relative to a single channel restrictor and the ability to continue forming particles if some channels are blocked or clogged. The benefits of using an elongated exit for fluids include: 1) a more dispersed (axially) plume of aerosols which helps avoid problems of aggregation of particles

25   after formation and 2) a better mixed fluid.

The multichannel restrictor may be used to form particles of various substances, including fluids; melts; solutions; supercritical fluids and solutions or suspensions of supercritical fluids and aqueous and/or organic solvents; emulsions; microemulsions; micelles;

30   reverse micelles and other substances into aerosols containing fine particles of solids (amorphous

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or crystalline). These aerosols may be dried using the methods described herein, or other methods known to the art. The multichannel restrictor may also be used in fire extinguishers, where free radical scavengers can be used in combination with removal of heat to smother a fire. An aerosol cloud of very finely divided water droplets containing radical scavenger has higher

5   surface areas and droplet suspension lifetime without sedimentation than larger water droplets prepared by conventional spray nozzles without CD_2.

EXAMPLES

Materials

10   Microcrystalline egg white lysozyme, crystallized (3x), dialyzed in water and lyophilized was purchased from Sigma Chemical Co. lot# 53H7145. Lactate dehydrogenase was obtained as an ammonium salt suspension, purchased from Sigma Chemical Co. (isolated from rabbit muscle, M_4 isoenzyme lot# 95H9550) and from Boehringer-Mannheim (isolated from porcine heart, M_4 isoenzyme, Batch # 84895527). Sucrose and mannitol were purchased from Pfanstiehl

15   Laboratories and used without further purification. Polyoxyethylene (20) sorbitan monolaurate (Tween 20) and buffer salts were purchased from Aldrich Chemical and used without further purification. Carbon Dioxide (SFE grade, siphon tank) was purchased from Scott Specialty Gases.

20   Methods

Preparation of the Enzymes. Lysozyme solutions were prepared by the addition of the solid, previously lyophilized, material to the desired formulation. Lysozyme is a fairly robust protein that is not significantly damaged upon lyophilization or conventional spray drying. The lyophilized powder was allowed to slowly diffuse into solution at a temperature between 2 and

25   8°C. The LDH ammonium sulfate suspensions were dialyzed against 100 mM potassium phosphate (pH 7.5) at a temperature between 2 and 8°C for 12 to 24 hours. The resulting solution was then diluted to a concentration of 100 mg/mL of protein in water.

Nebulization and Bubble Drying System. The system used for supercritical CO_2-assisted

30   nebulization is summarized here briefly (diagram in Figure 1). An aqueous stream and a stream

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of supercritical or near critical carbon dioxide (T > 32°C, P = 1500 psi) were each delivered at a constant flow rate of approximately 0.3 mL/min into each of two legs of a low dead volume mixing tee (Valco) using a HPLC solvent delivery pump (Waters model M-6000A) for the aqueous stream and a syringe pump (ISCO Model 260D, set to deliver at a constant pressure of

5   1500 psi) for the carbon dioxide. The two streams, initially at room temperature, were heated to just above 32°C, by a thermocouple-controlled cartridge heater attached to the mixing tee. The resultant emulsion that formed inside the mixing tee was allowed to expand out of the third orifice of the tee which was fitted with a 50 μm inner-diameter 5 cm long fused silica pressure restrictor (Alltech). The rapid decompression of the supercritical fluid as it exited the pressure

10   restrictor, coupled with the explosive release of dissolved carbon dioxide from the aqueous solution caused the formation of very fine aqueous droplets containing some residual dissolved carbon dioxide. This aerosol was then directed into a custom-built drying chamber consisting of a 30 cm x 2 cm borosilicate glass tube fitted with 4 gas inlet ports at the top of the tube and a powder filtration apparatus (stainless steel Millipore filter holder, 0.2 μm pore size cellulose

15   acetate filter paper) followed by a cold trap and vacuum pump at the bottom of the tube. Heated dry nitrogen at a flow rate of approximately 15 L/min was added concurrently with the aerosol through the four gas inlet ports at the top of the drying tube. Additionally, the tube was heated externally with an infrared lamp to aid in the drying process and to keep water from condensing on the inner walls of the tube. The resultant temperature inside the drying chamber was

20   maintained below 70°C during nebulization, which was sufficient to cause rapid bubble drying.

Once the system was equilibrated by nebulizing and drying pure water for 10 to 15 minutes, an aqueous protein formulation (containing between 2 and 20% w/w total solids) was injected into the aqueous feed line via an HPLC-type injection port. This type of injection port

25   allows for very small volume aliquots (between 0.5 and 5 mL) of the aqueous protein formulation to be nebulized, dried and collected, which is especially useful when working with limited quantities of protein. The design of this system allowed for the collection of the dried powder under a constant purge of warm dry nitrogen, which was continued after completion of the experiment to remove excess water vapor from the drying chamber. The filter paper

30   apparatus was then isolated from the system and transferred to a dry nitrogen-purged glove bag,

where the powder was transferred into 1.5 mL microcentrifuge tubes and capped and stored in desiccators over conc. sulfuric acid until analysis. In some experiments, the vacuum pump was used to draw the water vapor, carbon dioxide, and nitrogen through the filter at pressures slightly below atmospheric (630 mm Hg in Boulder, CO at its mile-high elevation). In other experiments

5   the vacuum pump was eliminated and the drying was conducted at pressures slightly above ambient, with equivalent results (data not shown).

Static and Dynamic System pH Measurements

In order to simulate the conditions inside the low dead volume mixing tee in an

10   observable static system, unbuffered and buffered solutions mixed with Fisher pH indicating solution were filled into a high pressure chamber equipped with sapphire windows, a pressure transducer, thermocouple relay-controlled heating cartridges and a magnetic stirring mechanism. The contents of the cell were equilibrated at 35°C, with stirring, and pressurized to 1500 psi with CO_2. In order to determine the degree of increased acidity due to pressurization of the aqueous

15   formulations with carbon dioxide, a series of pH measurements was made on buffered and unbuffered solutions. In a second, more quantitative approach, buffered and unbuffered solutions were nebulized with the supercritical CO_2 system and collected as wet aerosols until the level of the aqueous solution in the collection vessel permitted a measurement by a pH probe.

20   Experimental Design

Samples for powder characterization were prepared with supercritical CO_2-assisted nebulization and bubble drying from the following aqueous lysozyme formulations: "buffer only" consisted of 4 mg/mL lysozyme and 100 mM potassium phosphate buffer (pH 7.0). "10% mannitol" consisted of 4 mg/mL lysozyme, 100 mM potassium phosphate buffer (pH 7.0) and

25   10% w/w mannitol. "10% sucrose" consisted of 4 mg/mL lysozyme, 100 mM potassium phosphate buffer (pH 7.0) and 10% w/w sucrose and "10% sucrose w/Tween" consisted of 4 mg/mL lysozyme, 100 mM potassium phosphate buffer (pH 7.0), 10% w/w sucrose and 0.01% w/w Tween 20 (polyoxyethylene (20) sorbitan monolaurate). Prior to nebulization, all formulations were filtered through 0.2 µm Nylon syringe filters to remove any particulates.

30   Approximately 2 mL of each formulation was injected into the nebulization system for each run,

except for the buffer only formulation, which was injected at 6 mL volume per run due to the low total solute content of this formulation (in an effort to collect enough powder per run for analysis). Each formulation was injected three times and each powder batch was collected separately to determine the run-to-run consistency of the system. Additionally, powders

5   containing LDH were prepared from aqueous formulations consisting of 0.1 mg/mL LDH and 100 mM potassium phosphate (pH 7.5), and with the following excipients: 10% w/w mannitol; 10% w/w sucrose; and 10% w/w sucrose with 0.01% w/w Tween 20. These concentrations refer to percentages in the aqueous solution before bubble drying; the concentrations in the dried powders become proportionately larger as the water is removed.

10

Size Exclusion Chromatography

The presence of soluble aggregates was determined by size exclusion chromatography

15   performed on a Dionex HPLC equipped with a model AD20 UV detector set to 280, a GP40 gradient fluid pump and a Spectra Physics autosampler. The stationary phase consisted of a 30 mm x 7.8 mm (i.d.) TosoHaas TSK-Gel $G3000SW_{xl}$ column of 5 mm silica beads with a pore size of 250 Å. The isocratic mobile phase consisted of 100 mM potassium phosphate buffer (pH 7.0). Prior to injection, powder samples were rehydrated to the original, pre-dehydrated, total

20   solids weight to total solution weight ratio with deionized water. The rehydrated samples were centrifuged and loaded into the autosampler in septa-capped HPLC vials. The flow rate was set to 0.5 mL/min, and approximately 10 mL of each sample was injected onto the column. Horseradish peroxidase, myoglobin, BSA, and ovalbumin were used as molecular weight standards. Each sample was injected three times and an average of each peak area was

25   determined.

Scanning Electron Microscopy

Powders were examined using an ISI-SX-30 and a Jeol JSM-6400 scanning electron microscope (SEM) operating at an acceleration voltage of 30 kV. Samples were adhered to

30   aluminum stubs using carbon tape and were gold sputter coated prior to analysis.

Thermal Analysis

Differential scanning calorimetry (DSC) was performed on a Perkin-Elmer DSC-7. Powders (5 to 10 mg) were loaded into anodized aluminum pans and hermetically sealed under nitrogen in a humidity-controlled atmosphere (<2% relative humidity). Each sample was rapidly cooled to -20°C, held at -20°C for 10 min and then heated to 200°C at a heating rate of 10°C/min. T_g is the glass transition temperature (glass → liquid). T_m is the melting temperature of solid → liquid.

X-ray Powder Diffraction

Powder X-ray diffraction was carried out on a Sintag PADV system equipped with a CuK_a source (1 = 1.54056 Å) operating at a tube load of 40 kV and 25 mA. To determine the presence or absence of crystallinity in the powders prepared by supercritical CO_2-assisted nebulization and bubble drying, samples were scanned over a range of 2θ from 5° to 50° at a scan rate of 0.02°/minute.

Moisture Analysis

Karl Fisher titrations were performed on a Mettler Karl Fisher automatic titrator. Samples for analysis were prepared in a nitrogen-purged glove box maintained below 1% relative humidity. Approximately 1 mL of anhydrous methanol or formamide was added to 25 mg of sample powder. The resulting suspension was sonicated for several minutes. 100 mL of the solution was injected into the coulometer and the moisture content of the powder was determined after subtraction of the background moisture content of the solvent in which the powders were suspended. Analysis of each powder sample was performed in triplicate.

Infrared Spectroscopy

Infrared spectra were obtained on a Bomem PROTA infrared spectrophotometer. Lyophilized powders (ca. 0.5 mg protein) were mixed with 300 mg anhydrous KBr and

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compressed into a pellet with a 13 mm evacuable die. The pellets were placed directly into the nitrogen purged sample chamber of the spectrophotometer. Aqueous solutions of native protein (20 mg/mL) were placed in a sample cell containing CaF_2 windows separated with a 6 mm Mylar spacer. An average of 128 scans was collected with 4 cm^{-1} resolution in the 4000 - 900 cm^{-1}

5   range and Fourier transformed. For aqueous samples, the spectra of liquid water and water vapor were subtracted from the protein spectra of the protein solutions according to previously established criteria. (Dong, A. et al. (1990), "Protein secondary structures in water from second-derivative amide I infrared spectra," Biochem. **29**:3303-3308; Dong, A. and Caughey, W.S. (1994), "Infrared methods for study of hemoglobin reactions and structures," Methods Enzymol.

10   **232**:139-175; Dong, A. et al. (1995), "Infrared Spectroscopic Studies of Lyophilization -- and Temperature -- Induced Protein Aggregation," J. Pharm. Sci. **84**:415-424.) The second derivative for the Fourier transformed spectra was calculated and a Savitzky-Golay smoothing of the data was applied with a seven-point convolution window to reduce possible white noise.

15   Lysozyme Enzymatic Activity Assay

A bacterial suspension of Micrococcus lysodeikticus (Sigma lot #38H8619) at a concentration of 0.25 mg/mL in 67 mM phosphate buffer (pH 6.6) was prepared. The lysozyme solutions were diluted to 4 mg/mL enzyme with the phosphate buffer. The reaction mixture consisted of 2.5 mL of the cell suspension and 0.1 mL of the enzyme dilution. The enzymatic

20   activity was proportional to the rate of the decrease in turbidity of the cell suspension, which was measured spectrophotometrically as a linear decrease in absorbance at 450 nm for 2 min.

LDH Enzymatic Activity Assay

LDH activity was measured at 25°C in a 2 mL reaction mixture consisting of 25 mM

25   tris(hydroxymethyl)aminomethane/ tris(hydroxymethyl) aminomethane hydrochloride (Tris/Tris HCl, pH 7.5), 100 mM KCl, 2 mM pyruvate (Sigma, lot #126H10981), and 0.15 mM NADH (Sigma lot #027H78191). The LDH preparation (10 mL) was added to the reaction mixture, the cuvette was inverted 3x and the absorbance decrease at 340 nm was monitored with a spectrophotometer. Activity was measured immediately prior to supercritical CO_2-assisted

30   nebulization and immediately after rehydration of the dried powders and reported as a percentage

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of initial activity. Note in Figure 11 the LDH activity is greater than 100% of the activity of the initial aqueous solution for certain compositions.

Static and Dynamic pH Measurements with and without Buffers

5   The question of whether the acidification occurring when CO_2 is dissolved in water could be avoided by the use of buffers was studied. The solubility of CO_2 (approximately 2 mole % at 35°C) in water at 1500 psi causes an increase in acidity in unbuffered solutions. In the absence of buffers, an acidic environment could potentially denature proteins. For these reasons, a static system was used to simulate the conditions within the mixing tee in order to observe the extent

10   of the increase in acidity of an aqueous solution without buffering. pH sensitive indicator dyes were added to the aqueous solution prior to pressurization with carbon dioxide to qualitatively monitor the change in acidity. As expected, the pH rapidly decreased below the indicator range (pH < 4) immediately upon pressurization. The capability to neutralize this effect was estimated with this static system for the following buffer salts: monobasic/dibasic potassium phosphate

15   (pH 7), Tris/Tris HCl (pH 7.2), citric acid/sodium citrate (pH 5.5), and acetic acid/sodium acetate (pH 5). The buffering capacity for all solutions tested, measured as the ability of the buffer system to forestall the color change of the indicator dye, leveled off at approximately 100 mM concentration (data not shown). To further quantitate these observations, the same buffers used in the static system were nebulized with the supercritical CO_2 system and collected as wet

20   aerosols. The pH of each solution had to be measured immediately after collection as the value continued to increase due to carbon dioxide effervescence. The results of this experiment are presented in Figure 5 and are consistent with the static observations that 100 mM buffer will adequately neutralize the effects of dissolved CO_2 and avoid damage to proteins that are damaged by acidification.

25

Fine Powder Characterization

In order to determine the surface characteristics of molecules produced by the method of the current invention, fine powders of albuterol sulfate, tobramycin sulfate, cromolyn sodium, rhDNase, lactose, and sodium chloride were prepared using the method outlined above.

30   Conditions for droplet formation were CO_2 pressure: 1500 psi, 0.3 mL/min flow rates, CO_2 and

H₂O concentrations were about 10% (wt/vol), tube inlet ~70°C, tube outlet ~50°C, 50 mm, 5 cm fused silica restrictor, 0.2 mm cellulose acetate filter paper.

Figure 6 shows electron micrographs of representative dry powders prepared using the method of this invention. Figure 6A is a transmission electron microscopy (TEM) image of dried sodium chloride particles prepared from the method of the invention using a 20% aqueous sodium chloride sample. Figure 6B is a scanning electron microscopy (SEM) image of the same sodium chloride solution after drying by the method of this invention. Figure 6C is a TEM image of mannitol. Figures 6D and 6E show tobramycin sulfate particles (6D) and 1% tobramycin sulfate in lactose particles (6E) prepared by the method of this invention on a 10% (total solute mass/volume) aqueous solution. Figure 6F is an image of albuterol sulfate particles produced by the method of this invention. Figure 6G is an image of cromolyn sodium particles produced by the method of the invention.

Dry powders were initially prepared by CO₂-assisted nebulization and bubble drying from the aqueous lysozyme formulations described in Materials and Methods. Visually, the collected samples were all very fine white powders with very little run-to-run variability in the quality of the powders. All powders were hygroscopic in moist air, but were free flowing and easily handled in a low-moisture environment. Electron micrographs of representative dry powder protein formulation are shown in Figure 7. "Buffer only" powder (A) 10% mannitol (B), 10% sucrose (C), and 10% sucrose with 0.01% Tween 20 (D). Note that when Tween is used, (see Figure 7D), a powder with less agglomerated particles and more distinctly spherical, individual particles is formed. This may be a direct or indirect effect from the addition of surfactant Tween 20. Without wishing to be bound by any particular theory, it is believed that the detergent aids in dehydration during processing. All powders produced had significant portion of particles well within the 1 to 3 mm diameter inhalable size range. The morphology of the powders prepared from the buffer only formulation and the formulation containing 10% mannitol appear to be significantly less spherical and smooth than the powders prepared from the formulations containing sucrose. The addition of Tween 20 to the sucrose solution had an effect to even further smooth out the surface of the dried particles. This has been observed by other

researchers for particles that were conventionally spray-dried and it is postulated that the addition of a surface active agent to the aqueous formulation has an effect to reduce surface turbulence during the dehydration process.

5   The micrograph of the 10% mannitol formulation (Figure 7B) indicates a more crystalline material as seen by the multi-faceted particles.

Moisture Analysis

Karl Fischer moisture analysis on lysozyme powders dried by the method of the

10   invention also indicated low moisture contents: the moisture content of samples of 4 mg/mL lysozyme with 10% (percentage in initial aqueous solution) mannitol was $0.6 \pm 0.05\%$; the moisture content of powders of 4 mg/mL lysozyme with 10% sucrose was $1.18 \pm 0.02\%$ and the moisture content of powders of 4 mg/mL lysozyme with 10% sucrose and 0.01% Tween was $1.25 \pm 0.07\%$.

15

The Karl Fisher titration results performed on each LDH powder prepared by supercritical CO_2-assisted nebulization and bubble drying indicate that quite low moisture contents are attainable for this new process: the moisture content of the powder prepared from the buffer only aqueous formulation was $0.83 + 0.06\%$ w/w, $1.3 + 0.01\%$, from the 10%

20   mannitol formulation, $1.4 + 0.08\%$ form the 10% sucrose formulation, and $3.25 + 0.03\%$ from the formulation containing 10% sucrose and 0.01% Tween 20. The collected sucrose powders were very hygroscopic, and had to be handled in a gloved bag under an atmosphere of dry nitrogen. Other sugar powders, e.g., trehalose, are less hygroscopic.

25   XRD and DSC

Because the crystallization of excipients can have dramatic effects on the solubility and stability of solid pharmaceutical formulations, the ability to form crystalline and/or amorphous powders with supercritical CO_2 assisted nebulization was investigated. The strong tendency of mannitol to crystallize during freeze-drying and to form crystalline powders upon spray drying is

30   well known. Therefore, mannitol was chosen as an excipient to determine the capability of the

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nebulization system to form crystalline powders, although this excipient was not expected to stabilize the model proteins to a significant degree. While crystallinity is a desired attribute for excipients for excipients used as bulking agents for solid pharmaceuticals, excipients that tend to crystallize during processing offer very little protection to labile protein drugs during

5   dehydration and subsequent storage in the dried state. The powder diffraction data for a lysozyme sample prepared from the 10% mannitol formulation appears in Figure 8 for samples prepared by supercritical CO_2-assisted nebulization. Aqueous formulations contained 4 mg/mL lysozyme, 100 mM phosphate buffer at pH 7.0 and were nebulized together with 10% mannitol (A) or 10% sucrose (B). Final approximate solid weight percentage in the bubble dried powders:

10   4% protein, 16% buffer and 80% sugar. The diffraction data of the powder prepared from the 10% mannitol solution indicates good crystallinity within the sample and appears to be a mixture of the a and b polymorphs of mannitol. This diffraction pattern is consistent with the powder diffraction pattern obtained for pure D-mannitol as received from the vendor (not shown).

15   DSCs for mannitol as received, mannitol spray dried with lysozyme, sucrose spray dried with lysozyme and sucrose and lysozyme are not shown. The DSC of a sample of mannitol as received showed a peak from 157.25 to 177.56°C; onset 156.10°C. T_m was 159°C; 300.47 J/g. PSC of a sample of mannitol spray dried with 4 mg/mL lysozyme showed a peak onset from 77.63 to 146.48°C. $T_m = 154.7$°C; 134 J/g uncorrected. DSC of sample of sucrose with 4

20   mg/mL lysozyme showed T_g from 21.93 to 47.12°C with onset 40.25°C. $T_g = 43.05$°C and 1.51 J/g deg uncorrected. DSC of sucrose with Tween and 4 mg mL lysozyme showed T_g from 28.57 to 56.99°C; onset 45.36°C; 1.00 J/g deg; $T_g = 50.95$°C.

        The X-ray powder diffraction data of the sucrose formulations (Figure 8B), indicate a

25   more amorphous nature confirmed by the glass transition observed in the DSC thermograms. The ability to form amorphous glassy powders below the glass transition temperature is an important capability of the disclosed system, as significant evidence has been found recently linking protein stabilization with vitrification (glass formation) (Crowe, J.H et al. (1998), "The Role of Vitrification in Anhydrobiosis," Annu. Rev. Physiol. 60:73). Differential scanning

30   calorimetry of the sample indicated the presence of an endothermic event at 154.7°C, which is

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consistent with the melting temperature of crystalline D-mannitol (169°C). The depressed T_m is probably due to the presence of water (approximately 1% as measured by Karl Fisher titration) in the powder.

5   Characterization of Rehydrated Solutions

Powders collected on the filter paper were transferred to small inert tubes and rehydrated with distilled, deionized water to the original wt/wt% (total solute mass/total solution mass). Activity assay of the rehydrated protein solutions compared to the original solutions indicated greater than 90% activity retention (or recovery) for all solutions sprayed. HPLC (size exclusion

10   Tosohaas column TSK3000SW$_{XL}$, 100mM KPO$_4$ pH = 7.0 elution buffer) indicated that the ratio of monomeric protein to dimeric aggregates remained constant after drying and rehydration (aggregate formation to produce dimers, trimers and eventually insoluble aggregates is a common protein degradation pathway). HPLC data are not shown. The aggregate:monomer ratio was calculated to be approximately 0.21% ± 0.02% for the initial lysozyme formulations

15   and for all rehydrated powders.

Researchers believe that one important method for stabilizing a protein in the solid state is to provide the protein with a matrix in which native protein structural conformation can be, at least somewhat, preserved upon dehydration (Chang, B.S. et al. (1996), "Physical Factors

20   Affecting the Storage Stability of Freeze-Dried Interleukin-1 Receptor Antagonist: Glass Transition and Protein Conformation," Arch. Biochem. Biophys. **331**:249; Allison, D.S. et al. (1998), "Effects of Drying Methods and Additives on Structure and Function of Actin: Mechanisms of Dehydration-Induced Damage and Its Inhibition," Arch. Biochem. Biophys. **358**:171). Dehydration-induced structural transitions have been shown to be inhibited by the

25   addition of certain stabilizers, such as sucrose, to the protein solution before processing (Prestrelski, S.J. et al. (1993), "Dehydration-induced Conformational Transitions in Proteins and Their Inhibition by Stabilizers," Biophys. J. **65**:661). Second derivative Fourier-transformed infrared spectroscopy (Carpenter, J.F. et al. (1998), "Application of Infrared Spectroscopy to Development of Stable Lyophilized Protein Formulations," Eur. J. Pharm. Biopharm. **45**:231) in

30   the amide I region (1600 - 1700 cm^{-1}) was used to probe the conformational structure of the dried

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lysozyme powders. Representative results are shown in Figure 9. Samples of (A) lysozyme in the native aqueous state; (B) lysozyme containing 10% sucrose and 0.01% Tween 20; (C) lysozyme with 10% sucrose; (D) lysozyme with 10% mannitol; and (E) lysozyme with buffer only were dried at 70°C. The amide I region shows characteristic C=0 stretching frequencies for

5   different types of secondary structures. Interestingly, while enzymatic activity was almost fully retained upon rehydration of all powders, the dried lysozyme powders showed markedly different protein conformations in each formulation. When compared to the native lysozyme liquid infrared spectrum, the formulation without any excipient showed the largest deviation from native conformation, the 10% mannitol formulation showed somewhat less deviation from

10   the buffer only formulation, while the 10% sucrose formulation showed even less deviation from the native structure. The 10% sucrose plus Tween 20 formulation showed the most native-like structure of all the powders. These results indicate that lysozyme was not irreversibly damaged by our nebulization and drying process. The protein structure, however, showed significant deviation from the native conformation in the dry powders. The recovery of activity seems to

15   indicate the ability of lysozyme to recover its native conformation upon rehydration. The conformational transitions observed in the drying process can be inhibited by the addition of disaccharide stabilizers such as sucrose.

     Without wishing to be bound by theory, it is believed that sugars hydrogen bond to the

20   protein in place of water molecules, thereby reducing conformational changes in the protein structure upon dehydration. It is believed that surfactants reduce the amount of stress at the air-water interface that the protein is subjected to by competing for the available air-water interface regions with the protein. Surfactants added to the protein formulation are believed to prevent aggregation of the protein molecules.

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Lysozyme Activity Assay

     Enzymatic activity of the lysozyme powders rehydrated to their original total solute % (w/w) was measured immediately after rehydration. These results (Figure 10) are reported as a percentage of the initial activity that was independently measured immediately before

30   supercritical CO_2-assisted nebulization and bubble drying. Results are an average of at least

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three measurements, and error bars represent one standard deviation. Analysis of lysozyme powders indicated greater than 90% recovery of initial activity for all formulations. The observed recovery of activity is an indication of the reversibility of the structural change in this protein upon dehydration and rehydration. Thus, although lysozyme is severely unfolded upon

5   dehydration during supercritical CO_2-assisted nebulization in the absence of amorphous stabilizers, it can readily refold upon rehydration, and, thereby, recover much of its original biological activity.

LDH Activity Assay

10   In order to further examine the nebulization process, studies were performed using a significantly more labile enzyme, lactate dehydrogenase (LDH, Sigma, L-5762). Data are shown in Figures 10 and 11. LDH is known to be damaged by conventional drying processes (Adler, M. and Lee, G. (1999), "Stability and Surface Activity of Lactate Dehydrogenase in Spray-Dried Trehalose," J. Pharm. Sci. **88**:199). LDH was dialyzed against the 100 mM potassium phosphate

15   buffered to pH = 7.5 at room temperature for at least 12 hours. Protein concentration was kept constant at 100 µg/mL. The LDH formulations were dehydrated using the same conditions used for bubble drying the lysozyme formulations. The powders prepared from the LDH solutions appeared visually identical to the powders containing lysozyme (data not shown).

20   Dry powders comprising LDH were produced from formulations containing 10% mannitol, 10% sucrose and 10% sucrose plus 0.01% Tween 20, along with LDH (100µg/mL) and potassium phosphate buffer (100mM, pH = 7.5). The activity of the rehydrated powders was compared to the original formulations before nebulization and drying.

25   Four different formulations were prepared:

Buffer only
0.1 mg/mL LDH
100 mM KPO_4 (pH 7.5)

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<u>10% Mannitol</u>

0.1 mg/mL LDH

100 mM KPO_4 (pH 7.5)

100 mg/mL mannitol

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<u>10% Sucrose</u>

0.1 mg/mL LDH

100 mM KPO_4 (pH 7.5)

100 mg/mL sucrose

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<u>10% Sucrose with Tween</u>

0.1 mg/mL LDH

100 mM KPO_4 (pH 7.5)

15   100 mg/mL sucrose

0.1 mg/mL Tween 20

The LDH catalytic activity recovery for these formulations is shown in Figure 11. LDH, nebulized and dehydrated without any added excipients, recovers only 15% of its original

20   activity after rehydration. Interestingly, the same solution collected as a wet aerosol retained 87% of its original enzymatic activity. This result indicates that most of the damage experienced by the protein occurs in the drying process after the aqueous solution/CO_2 emulsion is ejected from the restrictor tip. In other words, CO_2-assisted nebulization is not as harsh as the drying of micro-droplets and micro-bubbles in nitrogen at 70°C. The irrecoverable damage experienced

25   during the dehydration process was partially prevented by the addition of mannitol, and to a greater degree with sucrose. Activity loss was almost completely prevented by the addition of sucrose and Tween 20. These results are as expected from the structural analysis of the lysozyme formulations as LDH, a more labile, tetrameric enzyme is not expected to have the same ability to refold upon rehydration as lysozyme. As a result, activity was better preserved

when LDH was dried in the presence of sucrose as it is most likely that the protein's structure was preserved.

Figure 12 compares recovery of activity upon rehydration for varying percentages of mannitol, sucrose and (sucrose + Tween (0.01%)). Note that the percent recovery of initial activity shows the most dramatic change with concentration of sugar when Tween is used in the formulation.

In general these results indicate that the damage experienced by LDH during the supercritical CO_2-assisted nebulization and bubble drying can be inhibited by the addition of a sugar stabilizer and even further with the combination of 10% sucrose and 0.01% surfactant, Tween 20. The formulated protein appears to be successfully protected throughout the dehydration process experienced during supercritical CO_2-assisted nebulization and bubble drying.

The mechanism(s) by which Tween 20 increased protection in the presence of sucrose during supercritical CO_2-assisted nebulization and bubble drying is uncertain at this point. Tween 20 may protect the protein by saturating surface sites at the air-liquid interface thereby preventing surface denaturation. The air-liquid interface is expected to be significant for this process due to the high surface area micro-droplets produced by supercritical CO_2-assisted nebulization. Additionally, the surfactant may offer protection by saturating hydrophobic sites on the surface of the protein, which are potential sites for aggregation. Furthermore the surfactant may foster protein refolding during rehydration. Whether Tween 20 is protecting LDH by one or more of these mechanisms during nebulization and bubble drying is undetermined at this point. This protein protection effect, combined with the improved particle separation and smoothness noted in sucrose formulations containing Tween 20, clearly document the potential benefits of including this surfactant in formulations dried by supercritical CO_2-assisted nebulization and bubble drying. It was also noted in some experiments that the enzymatic activity of aqueous lysozyme after compression with CO_2, decompression, and bubble drying was apparently greater, as indicated by enzymatic analysis, than the initial aqueous

solution before compression at 2000 psi (see Fig. 12). It has been demonstrated by St. John, Carpenter and Randolph that protein refolding is facilitated by pressurizing aqueous solutions at 20,000 psi ("High Pressure Fosters Protein Refolding from Aggregates at High Concentrations," Proc. Natl. Acad. Sci. **96**:13029, 1999). At this time, the cause of the improved activity is not

5 known. Although Applicant does not wish to be bound by theory, treating aqueous solutions by compression with CO_2 with a surfactant may produce apparent improved activity due to the surfactant, the moderate pressure, the presence of carbon dioxide, or sugar, or by other mechanisms.

10 Although the description above contains many specificities, these should not be construed as limiting the scope of the invention, but as merely providing illustrations of some of the presently preferred embodiments of this invention. For example, many fine dry powders of many different substances can be used in the methods of the invention; other supercritical or near-critical fluids may be used than specifically exemplified; and additives other than those

15 specifically exemplified may be used. The scope of the invention should be determined by the appended claims and their legal equivalents rather than by the examples given.

Each reference cited in the present disclosure is incorporated by reference herein to the extent not inconsistent with the disclosure herein.

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